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Physicochemical, antioxidant, DNA cleaving properties and antimicrobial activity of fisetin-copper chelates

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ABSTRACT

Fisetin (3,3′,4′,7-tetrahydroxyflavone) metal chelates are of interest as this plant polyphenol has revealed broad prospects for its use as natural medicine in the treatment of various diseases. Metal interactions may change or enhance fisetin biological properties so understanding fisetin metal chelation is important for its application not only in medicine but also as a food additive in nutritional supplements. This work was aimed to determine and characterize copper complexes formed in different pH range at applying various metal/ligand ratios. Fisetin and Cu(II)-fisetin complexes were characterized by potentiometric titrations, UV–Vis (Ultraviolet–visible spectroscopy), EPR, ESI-MS, FTIR and cyclic voltammetry. Their effects on DNA were investigated by using circular dichroism, spectrofluorimetry and gel electrophoresis methods. The copper complex with the ratio of Cu(II)/fisetin 1/2 exhibited significant DNA cleavage activity, followed by complete degradation of DNA. The influence of copper(II) ions on antioxidant activity of fisetin *in vitro* has been studied using DPPH, ABTS and mitochondrial assays. The results have pointed out that fisetin or copper complexes can behave both as antioxidants or pro-oxidants. Antimicrobial activity of the compounds has been investigated towards several bacteria and fungi. The copper complex of Cu(II)/fisetin 1/2 ratio showed higher antagonistic activity against bacteria comparing to the ligand and it revealed a promising antifungal activity.

1. Introduction

Flavonoids are a group of compounds of plant origin, derived from polyphenols. Over the decades, they have gained special attention because of the numerous biological properties. They show both *in vitro* and *in vivo* antioxidant, anti-inflammatory, anti-cancer, anti-atherosclerotic activities and prevent the development of neurodegenerative diseases [1–6].

It has been shown that flavonoids have the ability to combine with metal ions to form chelate complexes that exhibit greater biological activity than the free flavonoids [7–10]. Among these ions, copper ions are particularly interesting. Copper is a trace element essential to the life of many organisms because it is involved in the processes of photosynthesis and respiration. It is present in the active centers of many enzymes [11]. Copper ions play an important role in biological systems and wherefore they are very popular in the area of biomedical research and bioinorganic chemistry. Therefore, copper was the subject of our investigation to determine the chelating properties and biological activity of fisetin. Fisetin (3,3′,4′,7-tetrahydroxyflavone) (Scheme1) is a naturally occurring bioactive plant compound of immense importance as potentially useful therapeutic drug for various free radical mediated as well as other diseases [12–14]. It is also added to nutritional supplements at very high concentrations and has a variety of pharmacological effects [15–17]. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by free radicals and by chelating metal ions [18]. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules [19]. The flavonoids, as natural metal chelators may suppress the Fenton response and lipid peroxidation [20]. Research on copper complexes with natural products or bioactive ligands is very helpful in developing new

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medicines, based on these complexes [21]. Flavonoid–metal complexation reactions are very complicated due to many variables participating in and affecting the final outcome. Although extensive experimental and theoretical investigations have been performed, there are still different perspectives on mechanisms, sites chelation orders, structures, stoichiometries and stabilities of the complexes formed, especially in solutions [19,22–24]. Studies in solution are indispensable, because biologically active substances act in the cell compartments with different water contents, and therefore the information about the acid-base properties and the process of coordinating metal ions give an idea of which chemical form of biomolecule may be involved in biochemical processes in the cells.

This paper is devoted to research regarding the complexation of copper(II) ions with fisetin in methanol-water solutions, interactions of complexes with calf thymus DNA or plasmid DNA, their antioxidant action *in vitro* and antimicrobial activities. Different analytical methods adequate to characterize physicochemical properties of fisetin and its copper complexes (potentiometric titration, spectroscopic techniques: UV–Vis, EPR, ESI MS, FTIR and cyclic voltammetry) and their effects on DNA (spectrofluorimetry, circular dichroism and gel electrophoresis) were applied. Antimicrobial activity of the compounds has been evaluated towards several bacteria: *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella* Typhimurium, *Salmonella* Enteritidis and fungi: *Geotrichum candidum*, *Alternaria alternata*, *Aspergillus ochraceus*, *Penicillium* sp.

2. Experimental

2.1. Materials

The racemic fisetin (3,3′,4′,7-tetrahydroxyflavone) presented in this work in abbreviation form as Fis, NaOH, KCl, KNO_3, $CuCl_2$, $Cu(NO_3)_2$, $CuSO_4{\cdot}5H_2O$, methanol (CH_3OH) and all other compounds were purchased from Sigma-Aldrich Co. All reagents were of analytical quality and were used without further purification. The Cu(II) stock solutions were prepared by dissolving anhydrous $Cu(NO_3)_2$ or $CuCl_2$ in the exact amount of HNO_3 or HCl. The metal concentration was determined by complexometric titration with EDTA. Accurate acid concentration in the Cu(II) stock solution was determined by pH-potentiometric titration. The fisetin stock solutions were determined by the Gran's method [25].

2.2. Synthesis of the solid complex $[Cu(H_3L)_2(H_2O)_2]{\cdot}3H_2O$

1.0×10^{-1} mmol of $CuSO_4{\cdot}5H_2O$ was dissolved in 15 mL of water and 2.0×10^{-1} mmol of fisetin were dissolved in 35 mL of MeOH. Then the solution containing the metal ion was added slowly to the solution of the ligand. A red-brown precipitate was immediately formed; the polycrystalline sample was filtered off, washed with cool water, and dried at room temperature. Yield: 65%; m.p. 322–325 °C. Elemental analysis was calculated for $C_{30}H_{28}CuO_{17}$ (724.09): C, 49.76; H, 3.90; Cu, 8.76 (%), found (%): C, 49.38; H, 3.94; Cu, 8.56. IR spectra in KBr (ν_{max}, cm^{-1}): 1614 (C═O), 1566 (C═C), 1503 (C═C), 1282 (C−O−C). UV–Vis spectra in DMSO (λ_{max}, nm (ε_{max}, $M^{-1}\,cm^{-1}$)): 687 (92); 498 (4035); 432 (11450); 368 (11500).

2.3. Physicochemical measurements

Elemental analysis (C, H and N) of $[Cu(H_3L)_2(H_2O)_2]{\cdot}3H_2O$ complex was carried out on a EuroVector 3018 analyzer. The metal content of the complex was determined using atomic absorption spectrometer: AAS GBC 932 Plus (GBC Scientific Equipment Ltd., Australia) with copper hollow cathode lamp. The melting point of the complex was determined by an Electrothermal 9200 microscopic melting point apparatus. Its infrared (IR) spectra (4000–600 cm^{-1}) were recorded with a JASCO FT/IR-480Plus spectrometer using KBr disks, electronic absorption spectra with a Jasco Uvidec 610 spectrophotometer and EPR spectra with a Bruker EMX spectrometer operating in the X-band (9.4 GHz) equipped with a HP 53150A frequency counter at 120 K.

Potentiometric measurements were carried out in methanolic–aqueous solution (40%/60% v/v) at 298 K with constant ionic strength (0.1 M KCl) by using an automated system Molspin pHmeter (Molspin Ltd., Newcastle-upon-Tyne, UK) equipped with a digitally operated syringe (the Molspin DSI 0.250 mL) controlled by a PC computer, using a Russel CMAWL/S7 semi-micro combined electrode. The titrations were done with carbonate-free NaOH solution of accurately known concentration (*ca.* 0.1 M). The concentrations of the base and HCl or HNO_3 solutions were determined by pH-potentiometric titrations. The electrode system was calibrated according to Irving et al. [26] and the pH-metric readings could therefore be converted into hydrogen-ion concentrations. The average water-ionization constant, pK_w was 13.78 ± 0.05 with methanol/water (40%/60%, v/v) as solvent. This value is similar to that presented in the literature (pK_w = 13.71) [27]. A slight difference results from the use of different measurement conditions as well as the assumptions made in the calculation. The samples were deoxygenated by bubbling purified argon for *ca.* 10 min prior to the measurements, as well as during the titrations. The pH-metric titrations were carried out in the pH range 2.0–12.0 and the initial volume of the samples was 2.0 mL. The ligand concentration was 1×10^{-3} M and the concentrations of Cu(II) were 1×10^{-3} M, 2×10^{-3} M for metal:ligand ratios 1:1, 2:1 or 2×10^{-3} M of the ligand and 1×10^{-3} M of Cu(II) for 1:2 ratio. The accepted fitting of the titration curves was always < 0.01 mL. The number of experimental points was within 100–150 for each titration curve. The reproducibility of the titration points included in the evaluation was within 0.005 pH units in the whole pH range examined. Protonation constants of the ligand and the overall stability constants of the complexes (β_{pqr}, where p, q and r represent the number of metal, ligand and proton in each of the $Cu_pL_qH_r$ stoichiometries, respectively) were evaluated by iterative non-linear least squares fit of the potentiometric equilibrium curves through mass balance equations for all the components, expressed in terms of known and unknown equilibrium constants using the computer program SUPERQUAD [28]. The value obtained for sigma (the root mean squared weighted residual), after refinement of the stability constants, was ≤ 1, which means that the data was fitted within experimental error. The equilibrium constants reported in this work were obtained from fittings that used three titration curves simultaneously (examples of titration curves are included in SI Fig. S1).

UV–Vis spectrophotometric pH titrations were carried out with solutions containing Fis and Cu(II) by using a Perkin-Elmer Lambda 11 spectrophotometer in the λ interval 200–900 nm using a quartz cell with a path length of 1 cm. The metal-to-ligand ratios were 1:1, 2:1 and 1:2, respectively in 40%/60% (v/v) of CH_3OH/H_2O. The same apparatus was used in DNA experiments and to investigate scavenging radical capacity.

High-resolution mass spectra (ESI MS) were obtained on a BrukerQ-FTMS spectrometer (Bruker Daltonik, Bremen, Germany), equipped with an Apollo II electrospray ionization source with an ion funnel. The mass spectrometer was operated in the positive ion mode. The instrumental parameters were as follows: scan range *m/z* 200–1200, dry gas–nitrogen, temperature 170 °C, ion energy 5 eV. Capillary voltage was optimized to the highest S/N ratio and it was 4500 V. The small changes of voltage (± 500 V) did not significantly affect the optimized spectra. The samples (metal/ligand in a 1/1 and 2/1 stoichiometry, $[ligand]_{tot} = 10^{-4}$ M) were prepared in 1:1 acetonitrile-water mixture at pH 3 and 7. The sample was infused at a flow rate of 3 μL/min. The instrument was calibrated externally with the Tunemix™ mixture (Bruker Daltonik, Germany) in quadratic regression mode. Data were processed by using the Bruker Compass DataAnalysis 4.0 program. The mass accuracy for the calibration was better than 5 ppm, enabling together with the true isotopic pattern (using SigmaFit) an unambiguous confirmation of the elemental composition of the obtained complex.

Cyclic voltammetry measurements were performed with an Autolab PGSTAT12 (Ecochemie) potentiostat/galvanostat interfaced with a PC under NOVA 1.10 software. All electrochemical tests were carried out in a single-compartment, three-electrode cell, at room temperature, under Ar atmosphere. The ligand concentration was 5×10^{-5} M, and the Cu(II) concentration was 1×10^{-4} M. The working electrode was a 3 mm diameter glassy carbon (GC) disk, an aqueous saturated calomel electrode (SCE) was the reference electrode, and a platinum wire was the auxiliary electrode. The working electrode was polished subsequently with 1 and 0.3 μm alumina powder and then rinsed with distilled water in an ultrasonic bath for 5 min before use. The experiments were carried out in CH_3OH/H_2O (40%/60% v/v) solvent mixture using 0.1 M $LiClO_4$ as supporting electrolyte or in acetate or phosphate buffer. The potential scan rate was equal to 100 mV s^{-1}.

2.4. Studies of free radical scavenging capacity

DPPH (2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) were used to establish and compare scavenging radical activity of fisetin and its copper complexes. DPPH radical scavenging activities of fisetin and its complexes of molar ratios Cu(II)/fisetin 1/1, 2/1 and 1/2 regarded as potential antioxidants were measured by already reported method. First the standard solutions of each antioxidant were prepared with different concentration (from 8×10^{-6} to 4×10^{-4} M) in methanol; 0.1 mL of it was added to 3.9 mL of the freshly prepared DPPH solution of 5.765×10^{-5} M in methanol. The decrease in absorbance at 517 nm is a measure of the reducing capacity of the flavonoid or the metal complex and it was followed during 30 min until the reaction reached a steady state (absorbance stabilized). The blank solution of DPPH was screened to estimate DPPH decomposition during the time of measurement. The exact DPPH initial concentration (C_{DPPH}) in the reaction medium was calculated from the calibration curve with the equation determined by the linear regression. The decrease in absorbance was converted into percentage by using the linear equation: $A_{517} = 13{,}565 \times C_{DPPH} + 0.00355$, $R^2 = 0.99978$. The decrease in absorbance was converted into percentage according to % $DPPH_{scavenging} = A / A_0 \times 100\%$ [29]. The relationship between % $DPPH_{scavenging}$ and antioxidant concentration is presented in Fig. S2. The measurements were performed at room temperature (25 °C) with the analyses carried out in triplicate. DPPH solution without test samples served as the control.

A mixture of ABTS and peroxydisulfate was prepared in water, and incubated at room temperature for 12–16 h. The product $ABTS^{+}\cdot$ ($\varepsilon_{734} = 15{,}000\ M^{-1}\ cm^{-1}$) was diluted to 50 μmol in 10 μmol phosphate buffer (pH 7.4). The reduction of $ABTS^{+}\cdot$ by antioxidants: fisetin or its copper complexes was monitored at $\lambda_{max} = 734$ nm. The decrease in absorbance at 734 nm is in accordance with the reduction of $ABTS^{+}\cdot$ ion. The experimental results demonstrated that the reaction with $ABTS^{+}\cdot$ was completed within 2 min. Trolox was used as an appropriate standard. An example of spectra registered for the complex with 1/1 Fis/Cu stoichiometry is included in SI as Fig. S3.

2.5. Mitochondrial studies

All experiments of isolation of rat liver mitochondria have been performed under the international rules “Guide for the Care and Use of Laboratory Animals”. Male Wistar rats weighing approximately 200–300 g were killed fast by decapitation after an overnight, and their livers were removed for immediate isolation of mitochondria. Mitochondria were isolated as described [30,31] with slight modification. Tissues were rinsed with saline, dried with filter paper, weighed and homogenized in a glass-teflon homogenizer with ice cold isolation medium containing 250 mM sucrose, 20 mM Tris-HCl and 1 mM EDTA, pH 7.2, at 4 °C. The homogenate was centrifuged at 600*g* for 10 min, and the supernatant was centrifuged at 8.500 g for 10 min at 4 °C. The obtained pellet was washed in buffer containing 250 mM sucrose, 20 mM Tris-HCl, pH 7.2 (at 4 °C). The protein concentration of 35–40 mg/mL was determined by the Lowry method [32]. *tert*-Butyl hydroperoxide (tBHP), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), calcium chloride dehydrate, thiobarbituric acid (TBA), trichloroacetic acid (TCA) were purchased from Sigma-Aldrich.

In order to test the effect of compounds on the antioxidant activity the initial pre-incubation of isolated rat liver mitochondria in the presence of 40 μM fisetin or the complex ($[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$) was carried out for 20 min at a temperature of 25 °C. After a preincubation to induce oxidative stress in isolated rat liver mitochondria *tert*-butyl hydroperoxide (tBHP) (1.5 mM) was added. The reaction mixture was incubated for 30 min at 25 °C. The protein concentration (15 mg/mL) in the mitochondria samples was determined by the method of Lowry et al. [32]. The concentration of reduced glutathione (GSH), total (TSH) and protein (PSH) thiols in mitochondria was determined spectrophotometrically by the method of Ellman [33] using the molar absorption coefficient $\varepsilon_{412} = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$. Mixed disulfides (GSSP) formed by glutathione and accessible sulfhydryl groups of mitochondrial proteins were determined by the method described by Rossi et al. [34]. The level of accumulated lipid peroxidation products (thiobarbituric acid-reactive substances, TBARS) was determined according to Stocks and Dormandy [35].

0.1 mL mitochondrial pellet was resuspended in 0.2 mL H_2O and subjected to three freeze-thaw cycles before measurements of mitochondrial enzyme activity [36]. The activity of glutathione peroxidase (GPx) in the mitochondrial pellet was measured by the method of Martinez et al. [37]. The activity of mitochondrial glutathione-*S*-transferase (GST) was determined employing the method of Habig et al. [38]. The absorbance measurements in all assays were performed using a spectrophotometer V-530 Jasco. The results were statistically analyzed using Statistics software 6.0. All results are expressed as arithmetic mean of four independent experiments ± SEM. A statistical significance of differences was determined by Student's unpaired *t*-test.

2.6. DNA experiments

Deoxyribonucleic acid sodium salt from calf thymus (CT DNA) was purchased from Sigma (#D3664). Absorption spectra titrations aiming to evaluate intrinsic binding constants (K_b) of Fis or complexes to CT DNA were carried out in Tris buffer (5 mM Tris-HCl, 50 mM NaCl, pH 7.2) at room temperature. The concentration of CT DNA was determined from the absorption intensity at 260 nm with a ε value of $6600\ M^{-1}\ cm^{-1}$. Absorption titration experiments were performed by varying the concentration of the CT DNA (0–25 μM) keeping the constant concentration (25 μM) of the ligand or complexes. All measurements were carried out after incubation at room temperature for 5 min. The stock solutions of fisetin and the complexes were prepared in 40%/60% (v/v) of CH_3OH/H_2O. The absorbance (A) was recorded after each addition of CT DNA. While measuring the absorption spectra an equal amount of DNA was added to the solution of the compounds and the reference solution to eliminate the absorbance of CT DNA itself, and Tris buffer was subtracted through baseline correction. The data were fitted to the Wolfe–Shimer equation [39] following the calculation procedure described in our earlier publications [40,41] to obtain the intrinsic binding constant of the compounds.

Fluorescence quenching experiments using a Hitachi Fluorescence Spectrophotometer F-2000 were carried out by adding increasing amounts of Fis or copper(II) complexes solutions (0–20 μM) to CT DNA–ethidium bromide (EB) system ($C_{EB} = 10$ μM, $C_{DNA} = 25$ μM, Tris-HCl (5 mM Tris-HCl, 50 mM NaCl, pH 7.2). DNA stock solutions were prepared by dissolving in TRIS-HCl buffer. The concentration of CT DNA (*ca.* 2.5 mM) was determined by UV–Vis absorbance using the molar absorption coefficient at 260 ($6600\ M^{-1}\ cm^{-1}$). The UV absorbance at 260 nm and 280 nm of the CT DNA solution gave a ratio of 1.9, indicating that the DNA was sufficiently free of protein. Emission

spectra were carried out in a 2 mL quartz cuvette with 470 nm excitation light, and emission was measured at 580 nm. The equilibration time was checked by measuring fluorescence spectra during 1 h (DNA: probe = 1:1), but no changes were observed, thus, the equilibration time was kept constant between measurements (5 min). Millipore water was used for the preparation of solutions. The solvent consisted of 40% (v/v) CH_3OH/H_2O was used for the preparation of the ligand and complex stock solutions (*ca.* 1.0 mM). UV–Vis absorption spectra were collected to correct the data for reabsorption and inner filter effects [42,43]. The concentrations were selected in order to have absorbance values below 0.2 at the excitation and emission wavelengths. The quenching extents of reported compounds were evaluated qualitatively by employing Stern-Volmer eq. $I_0/I = 1 + K_{SV}$ [C], where I_0 is the emission intensity in the absence of compound, I is the emission intensity in the presence of compound, K_{SV} is the quenching constant and [C] is the concentration of compounds. The K_{SV} values have been obtained as a slope from the plot of I_0/I *vs* [C].

Circular dichroism (CD) spectra were recorded with a Jobin Yvon CD6 spectropolarimeter at room temperature using quartz cell with a path length of 0.5 cm, 2 nm bandwidth and integration time of 1–2 s. Spectra were corrected for buffer signal and 25-point smoothing algorithm was performed with the Jobin Yvon CD6 Standard Analysis software. The CT DNA concentration used in the experiments was 9.4×10^{-5} M. Concentrations of fisetin and its copper(II) complexes varied from 1.88×10^{-5} M to 9.4×10^{-5} M. The samples were prepared in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, pH 7.2). The CD spectra of CT DNA were recorded with increasing concentrations of the ligand or copper(II) complexes after incubation at 37 °C. The CH_3OH effect on the DNA spectrum was evaluated in a distinct experiment and subsequently the percentage of CH_3OH was kept below 1.2% (v/v).

Electrophoresis experiments were carried out with pEGFP-C1 (4731 bp) DNA (BD Biosciences). The cleavage of pEGFP-C1 by fisetin and the complex with the stoichiometry of Cu(II)/Fis 1/2 ($Cu(H_3L)_2$) was accomplished by mixing in the following order: 1 μL of 5 mM Tris-HCl (pH 7.5 containing 5 mM NaCl) buffer, various concentrations (0; 25; 50; 100; 150; 200 μM) of the ligand or the complex and 1 μL of pEGFP-C1 (0.25 μg/μL; 10 mM Tris-buffer, pH 7.5). The solutions were incubated at 37 °C for 10 h. The reactions were quenched by addition of EDTA and bromophenol blue and the mixtures were analyzed by gel electrophoresis (0.5% agarose gel). Plasmid cleavage products were quantified and analyzed with the G-BOX Syngene system. The GeneTools software was used to complete gel documentation and analysis. Each concentration was assayed in triplicate in each experiment, and all experiments were repeated at least two times. The results were analyzed using one-way analysis of variance (ANOVA) $p \leq 0.05$.

2.7. Antimicrobial activity

Antimicrobial activities of $CuCl_2$, fisetin and $[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$ complex were tested against several bacteria and fungi. *In vitro* antibacterial activity studies were carried out against Gram-positive bacteria: strains of: *Listeria monocytogenes* (ATCC 19111, ATCC 19112 and ATCC 19115), *Enterococcus faecalis* (ATCC 29212-vancomycin sensitive strain and ATCC 51299-vancomycin resistant strain), *Staphylococcus aureus* (ATCC 29737, ATCC 23073 and ATCC 2773), *Staphylococcus epidermidis* (ATCC 12288, PCM 2480) and Gram-negative bacteria: *Salmonella* Typhimurium ATCC 14028, *Salmonella* Enteritidis ATCC13076. The bacteria were incubated on the Nutrient Agar (Merck, Germany) for 48 h at 30 °C for *Listeria* species and 48 h at 37 °C for other tested bacteria. The 24 h cultures were inoculated in Nutrient Broth (Merck, Germany) before use. The bacterial counts of the diluted cultures were corrected by adding isotonic NaCl solution to be within the range of 10^6–10^7 colony forming units (CFU).

In vitro antifungal activity studies were carried out against molds *Geotrichum candidum* 0511, *Alternaria alternata* 0409. The strains of fungi were obtained from Collection of Industrial Microorganisms of the Institute of Fermentation Technology and Microbiology ŁOCK 105, Lodz University of Technology. We used also two strains isolated by us from food: *Aspergillus ochraceus* and *Penicillium* spp. The environmental strains of fungi are frequently characterized by higher resistance than the collection strains. The molds and yeasts were incubated on the Sabouard Agar (Merck, Germany) for 72 h at 28 °C. The 78 h cultures were inoculated in Sabouard Broth (Merck, Germany) before use. The fungal spore suspensions (or yeast culture) were also corrected by adding isotonic NaCl solution to be within the range of 10^5–10^6 CFU.

Samples of test compounds were dissolved in DMSO to obtain a concentration of 5 mg/mL and were sterilized by filtration (filter pore width 0.2 μm; Sartorius). Paper disks (∅ = 5 mm) were impregnated with compound samples, to obtain a concentration of test compounds of 0.2 μM per disk, and the solvent was allowed to evaporate in the dark at room temperature. The diluted bacterial or fungal test culture (200 μL) was spread on sterile Mueller-Hinton Agar (Merck) plates for bacteria and Sabouard Agar (Merck, Germany) for molds before placing the sample. The impregnated paper disks were placed on the plates' surface. DMSO solution was used as a negative control at the concentration of 20 mg/mL (this concentration of DMSO did not inhibit the growth of microorganisms) [44]. Vancomycin (Oxoid) was used as positive control for Gram-positive bacteria, ampicillin (Oxoid) for Gram-negative bacteria, and nystatin (Oxoid) was used as positive control for the molds. After the inhibition, the diameters were measured. As a result, the final diameter of the disk was taken into account (subtracted). The experiments were repeated three times and results were expressed in average values and standard deviation. Assay was done in triplicate. The results were analyzed using one-way analysis of variance (ANOVA) $p \leq 0.05$.

2.8. Aggregation of bacterial strains

The aggregation assay was performed according to Ahmadova et al. [45] with some modifications. The overnight cultures of test strains (*Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Salmonella* species) were pelleted by centrifugation (8000*g*, 10 min, 20 °C), washed twice in sterile phosphate-buffer and saline (PBS, Sigma, pH 7.4) and resuspended in the same solvent to achieve an OD (optical density) of 0.5 at 600 nm. The 5.0 μL of $CuCl_2$, fisetin or $[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$ solutions were added to 0.995 mL of the bacterial suspension. The control sample was a suspension of bacteria in PBS. After 5 h incubation at 37 °C the absorbance of the upper part of cell suspension was measured at 600 nm. The percentage of aggregation was calculated as: *Aggregation* (%) = $[(A_0 - A_5) / A_0] \times 100$, where: A_0, initial absorbance; A_5, absorbance after 5 h. Assay was done in triplicate. The results were analyzed using one-way analysis of variance (ANOVA) $p \leq 0.05$.

3. Results and discussion

3.1. Fisetin

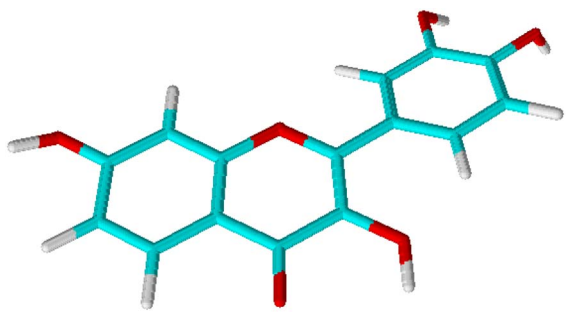
Acid dissociation constants pK_a are important parameters. The knowledge of their values allows predicting the presence of protonated or deprotonated species in a biological environment, very important in bioclinical and pharmacological studies. The role of flavonoids is related to their chemical structure. The hydroxyl moiety deprotonation has influence on their intrinsic antioxidant potential; deprotonation generally enhances the antioxidant action of the flavonoid [46]. Potentiometry is a very good tool to establish pK_a value of compounds of pharmaceutical and biological interest due to the accuracy and high precision. It can be used in pure water or aqueous-organic or even organic solvents [47,48]. When compound is sparingly soluble in water, the pK_a determination is commonly done in organic/water mixtures. It is the case of fisetin. We have chosen methanol/water mixture often used because of its lower polarity than pure water, but keeping a similar

Table 1
Ionization constants of fisetin and other flavonols for comparison.

Flavonol	Logβ_{HnL}	pK_1^s	pK_2^s	pK_3^s	pK_4^s	Ref.
Fisetin		C(7)−OH	C(4′)−OH	C(3)−OH	C(3′)−OH	
H_4L	37.38 (± 0.02)	7.51	8.63	9.71	11.53	[This work]
H_3L^-	29.87 (± 0.02)					
H_2L^{2-}	21.24 (± 0.02)					
HL^{3-}	11.53 (± 0.02)					
		7.31	8.59	12.11	13.85	[51]
3-Hydroxyflavone				9.40		[52]
Rutin		7.10	8.30	–	10.90	[53]

environment. The pK_a value at one unique methanol/water mixture is recommended because it is faster and simpler with respect to others for example to the classical Yasuda–Shedlovsky plot [49]. Therefore a mixed solvent with the ratio of 40%:60% v/v of methanol/water was used in these studies. Such a ratio is recommended by some authors as the least error-prone of mixed solvents because of a greater accumulation of information about the behavior of the glass electrode in this solvent [50]. Taking all these comments into account we decided to use potentiometric titration method to evaluate dissociation constants of fisetin phenolic groups. Ionization constants (pK) of fisetin determined in 40%:60% v/v of methanol/water solvent are reported in Table 1. Species distribution curves are presented in Fig. S4.

The chemical structure of flavonoids like fisetin (flavonol) (Scheme 1) implies a low acidity of the OH groups with respect to flavanones. Flavonols exhibit conjugation between the rings (B) and (A), which will facilitate the deprotonation of hydroxyl groups due to charge delocalization. The first deprotonation occurs in the ring A at C7 position, the second at C4′, the third at C3 and finally in ring B at C3′ position. Such an order is supported by experimental and computational studies [51]. It is found that the 3-OH fisetin radical has the lowest calculated BDE (bond dissociation enthalpies) value followed by the 3′-OH and 4′-OH fisetin radicals while the corresponding value of the 7-OH fisetin radical is predicted to be the highest [54]. The reverse order of the favorable hydration free energies ΔG^*_{hyd} was found for phenoxide anions derived from these groups: the 7-O^- > the 4′-O^- > the 3-O^- > the 3′-O^- respectively. Thus, for flavonols, the acidity of hydroxyl groups has been predicted to be similar [55]. Therefore, one can infer that releasing protons by phenol groups of fisetin will follow the same direction (Fig. 1). Ionization constants of fisetin determined in this work are similar to those found for other flavonols with the same ionizable OH groups (Table 1) [53]. The values of the pK_a illustrate that C(3)−OH and C(3′)−OH will dissociate above pH 9 while the groups located on other carbon atoms are sensitive to dissociation under conditions close to the physiological pH. The results lead to the conclusion that, dissociation constants determined for the C(7)−OH and C(4′)−OH should be particularly considered in evaluation of fisetin antioxidant activity in physiological pH conditions. Moreover, the results reveal that at pH 7.5 there is no clearly defined ionization state of fisetin molecule. Both forms neutral and ionized can coexist (Fig. S4). This may affect the mechanism of antioxidant activity of this flavonol *in vivo*.



Scheme 1. Fisetin [3, 3′,4′,7-tetrahydroxyflavone].

UV–Vis titration spectroscopy was used in order to investigate the pH effect on fisetin absorption bands (Fig. 2(A)). It was carried out in the pH range where neutral and ionized forms of the ligand were identified by potentiometry. The absorption spectrum of fisetin has three bands positioned at 248 nm, 319 nm and 361 nm (Fig. 2(B)). Absorption bands I and II belong to the cinnamoyl (B + C ring) and benzoyl (A + C ring) moieties. The bands are assigned to π-π* electronic transitions originating at these cinnamoyl and benzoyl systems [56]. Structural changes are evident in electronic spectra of fisetin at different pH values. The pH increase to 8.14 leads to the disappearance of the band II at 248 nm and the shift of the band I to higher wavelength (365 nm). In the pH range of 9.04–11.20 the latter peak undergoes a red shift (from 373 to 408 nm), and some disturbances in the shape of the band at 319 nm can be observed. The absorption of this peak diminishes progressively and a new peak at longer wavelength (333 nm) is seen at pH 11.20. This behavior is a consequence of the successive deprotonation as well as the coexisting neutral and ionic species in the solution (Fig. S4). By increasing the pH value, the bands strengthen intensities and shift slightly towards longer wavelengths due to the facilitated deprotonation caused by the stabilization of the anionic species [57]. The deconvolution of the experimental absorption spectra of fisetin reveals the bands that may correspond to the neutral and anionic species present in solution to various extents depending on pH (Fig. S5(A, B, C)).

3.2. Copper-fisetin complexes

Cu(II)-fisetin system has been studied by potentiometry and spectroscopic methods (UV–Vis, EPR, ESI MS) at different metal:ligand ratios 1:1, 2:1 and 1:2. Due to the fact that the precipitate is formed in a system containing two-fold excess of fisetin relative to Cu(II) ions, the fitting of potentiometric titration data and chemical speciation equilibria could not be performed (Fig. S1). However, this precipitate was isolated and analyzed (see Synthesis of the solid complex). The fitting of experimental data using SUPERQUAD program was only carried out in the systems with Cu(II):fisetin ratios of 1:1 and 2:1. The results are presented in Table 2. The stoichiometry and the values of overall stability constants of complexes were found out to be the same in both systems but some difference can be observed in species distribution curves (Fig. 3). Both mono- and dinuclear species with deprotonated ligand can be present in the solutions. As tetrahydroxyflavone, fisetin has two potential sites for chelation of metal cations: 3-hydroxy and 4-carbonyl group in the C ring and the catechol unit in the ring B. The order of chelating efficiency of flavonoid depends on charge on the chelating site. The binding energy for the metal atom to a single flavonoid molecule demonstrated that 3-hydroxyl and 4-carbonyl group is optimal chelation site, followed by 3′–4′ site [8,58–60]. The structure of quercetin 2,3-dioxygenase also supports the involvement of 3-OH and 4carbonyl coordination [61]. The studies of metal ion complexes with morin and its sulfonate derivatives indicated this group as chelating site as well [9,62,63]. Therefore, we believe that in acidic pH range, the

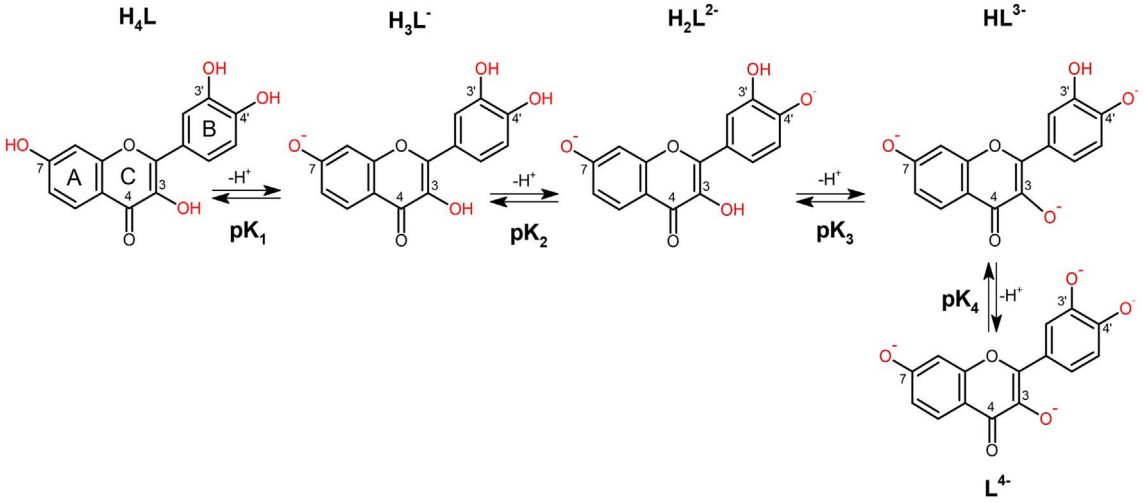


Fig. 1. Proposed scheme of deprotonation of fisetin in methanol/water solvent.

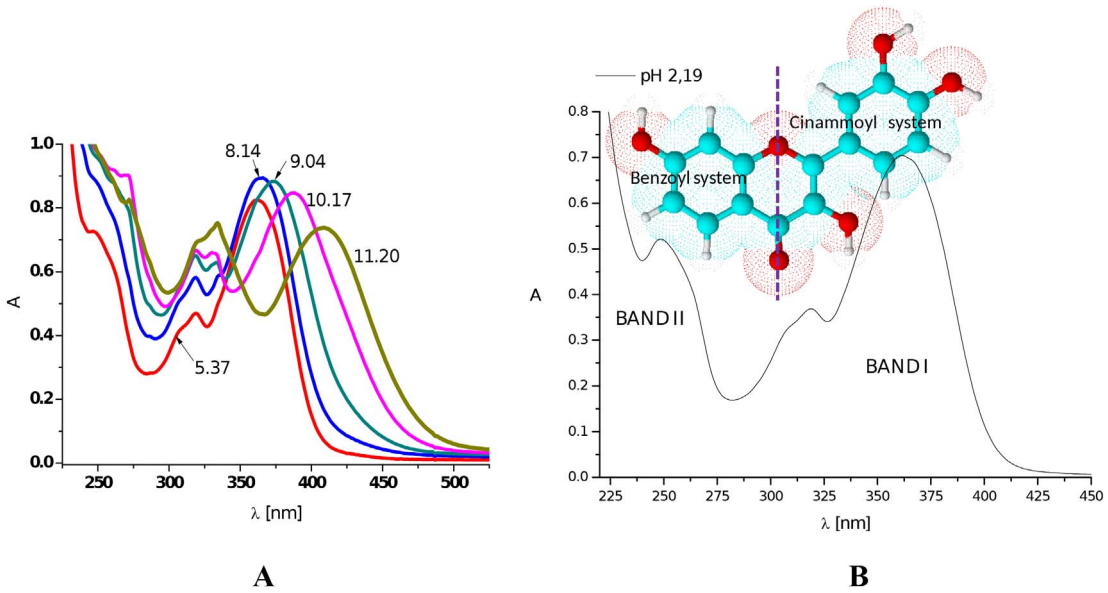


Fig. 2. (A) Titration electronic absorption spectra of fisetin in solution of 40%/60% v/v methanol/water and 0.1 M NaCl; $C_{fisetin} = 2.5 \times 10^{-5}$ M; (B) absorption bands of fisetin at pH 2.19.

Table 2
Overall stability constants ($\log\beta_{Cu_pH_rL}$) evaluated in the system of Cu(II)–fisetin at the ratios: 1:1 and 2:1. Standard deviations are given in parenthesis.

Species	Logβ
CuH_3L^+	36.51 (± 0.03)
CuH_2L	31.73 (± 0.03)
$CuHL^-$	26.59 (± 0.03)
CuL^{2-}	15.72 (± 0.04)
Cu_2L	25.63 (± 0.03)

first coordinating site for Cu(II) ions is the group situated in the C ring (Fig. 4). Above neutral pH, the next potential binding site of Cu (II) ions is a catechol moiety C(3′)—OH, C(4′)—OH in the ring B of fisetin [64]. It can be observed that the different protonated species appear one after the other with increasing pH region. The complexation process starts above a pH of 3. It is not surprising that in spite of the high pK_a values of fisetin ionizable groups, the protons are displaced at much lower pH range in the presence of copper(II) ions. The same process was observed for phenols and flavonoids under metal chelation [65]. The first CuH_3L^+ species has the [C(4)—O_{ket}, C(3)—O^-] donor atom set with 7–OH, 3′–OH and 4′–OH fully protonated. The next CuH_2L and $CuLH^-$ have the same donor atoms but they differ in the number of deprotonated phenolate groups (see Fig. 4). The formation of dimeric complex Cu_2L results from bonding of Cu(II) ions to two chelating sites [C(4)—O_{ket}, C(3)—O^-] and [C(3′)—O^- and C(4′)—O^-]. The CuL^{2-} forming in alkaline pH (above 11) can acquire the [C(3′)—O^-, C(4′)—O^-] coordination mode. The formation of complexes can be seen in UV–Vis spectra (Fig. 5). Red or blue shifts are observed, depending on metal/ligand ratio, with increasing pH values. It is caused by changes of conjugative effect when the complexes are formed to give a new ring. On addition of Cu(II) to a pH around 5, the fisetin long-wavelength band at 361 nm belonging to the cinnamoyl moiety (rings of B + C), loses intensity and shifts slightly bathochromically (Fig. 5(D)) giving rise to new absorption bands at 282 and 430 nm. It corresponds to complex formation (Fig. 3). The presence of copper(II) in fisetin solution in pH close to neutral produces the different results as in acidic solutions depending on molar ratio of ligand to metal (Fig. 5(E)). At the ratio 1:1 the band at 361 nm shifts hypsochromically ($\Delta\lambda$ = 5 nm) and the intensities of other bands are decreasing. The double excess of copper leads to a total disappearance of the fisetin long-wavelength

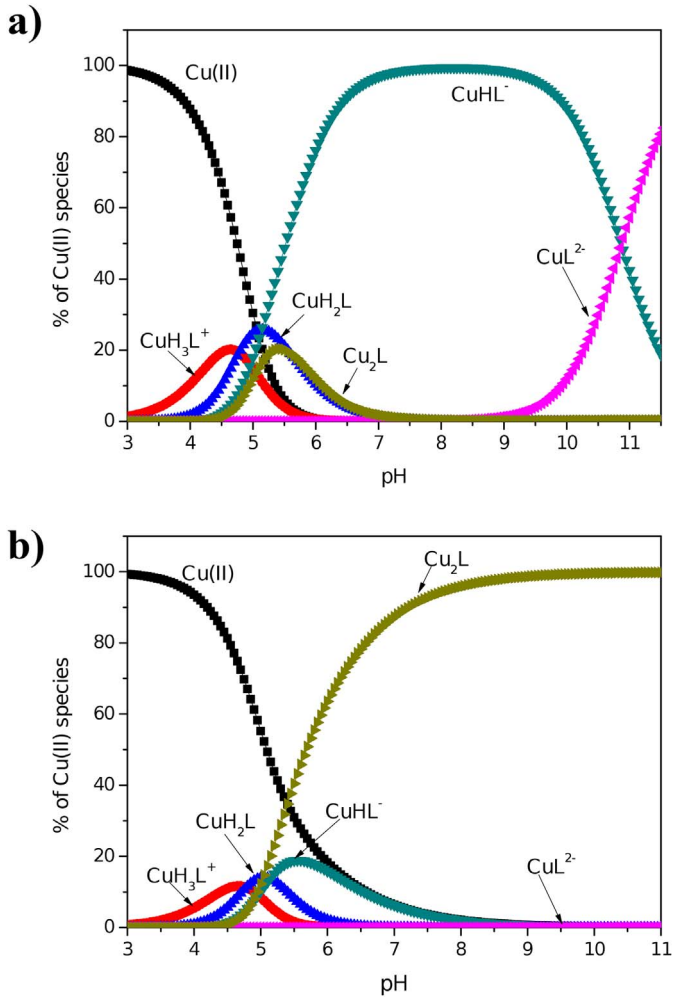


Fig. 3. Species distribution curves for the systems containing different ratios of Cu (II):fisetin a) 1:1 at $C_{Cu(II)} = 1 \times 10^{-4}$ M, $C_{fis} = 1 \times 10^{-4}$ M and b) 2:1 at $C_{Cu(II)} = 1 \times 10^{-4}$ M, $C_{fis} = 5 \times 10^{-5}$ M.

band and an increase of intensity of the band at 282 and a red-shift of the band 430 nm to 470 nm (the similar phenomenon was observed for quercetin [66]). In alkaline solutions the spectrum registered at a ratio 1:1 is similar to that of fisetin with very small shifts of the bands and lower intensities (Fig. 5(F)). On the other hand, a distinct change of fisetin spectrum can be observed upon addition of copper(II) to double excess. The bands characterizing fisetin at pH 9.05 are hypsochromically shifted (about $\Delta\lambda \approx 45$ nm). The spectral differences visualized at 1:1 and 1:2 fisetin:copper ratios indicate a formation of complexes with different stoichiometry at pH neutral and alkaline. The spectra recorded in visible region with increasing pH reveal a gradual disappearance of the band around 800 nm characteristic for copper(II) aqua ions and formation of a new band at around 550 nm (Fig. 5(C)). It can signify that fisetin is more effective chelator for Cu(II) ions in neutral pH region.

The stoichiometry of complexes was investigated by mass spectrometry. The data are included in Fig. S6. The peaks at *m/z* 413.3 and *m/z* = 371.1 correspond to $[2Cu(II) + L]^+$ and to an adduct CuNaL, respectively, where L is fisetin. These results clearly support the formation of complexes with stoichiometries CuL and Cu_2L indicated by potentiometry and electronic absorption spectroscopy.

The effect of EDTA addition to the system of Cu(II)–fisetin with 2:1 ratio was investigated in UV–Vis spectra. The studies were carried out at acidic and neutral pH in order to find out competitive effect of EDTA on a recovery of fisetin spectrum (Fig. 6). Adding EDTA to methanol solution of Cu(II)/fisetin 2/1 system at pH around 3.20 led to disappearance of a band at 428 nm characteristic for copper-fisetin chelate. The spectrum returned to its original position and characteristic absorbances for fisetin (Fig. 6(a)). It means that the recovery of fisetin is close to 100%. A different spectral appearance was obtained for phosphate buffer solution at pH 7.00 (Fig. 6(b)). Extent of recovery of fisetin spectrum is lower than at pH 3.20 although a spectral profile is maintained. In this both cases copper-fisetin complexes can be completely or partially destroyed. They are replaced by copper-EDTA chelates. The d-d band seen at 750 nm after addition of EDTA clearly supports their formation (see Fig. 6, the insets). These results suggest that at pH 7.00 with 2-fold excess of Cu(II) ions where Cu_2L complex is present (see Fig. 3(b)), the fisetin has not reverted to its original form but it has become very likely oxidized. It may be concluded that the oxidation of the free C(3)-hydroxy group has occurred during Cu^{2+} ion–fisetin interaction, and that the changes in band I suggest an additional effect on the hydroxy groups of B ring. Probable structural transformations of the oxidized fisetin may occur as it is shown in Scheme 2. These observations are consistent with the reported reducing properties represented by redox potential of phenoxyl radical/phenol couples $E_7(Q\cdot-/QH_2)$ [67], in that fisetin is an effective reducing agent with redox potential $E_7(Q\cdot-/QH_2)$ equal 0.33 V closely similar to those of quercetin (0.33 V) and myricetin (0.36 V) but much lower than other flavonoids.

Cyclic voltammetry (CV) experiments were carried out in order to investigate the evolution of the coordination process depending on the time and on the pH value. CV responses were recorded in solutions of CH_3OH/H_2O 40%/60% v/v and $LiClO_4$ 0.1 M or acetate or phosphate

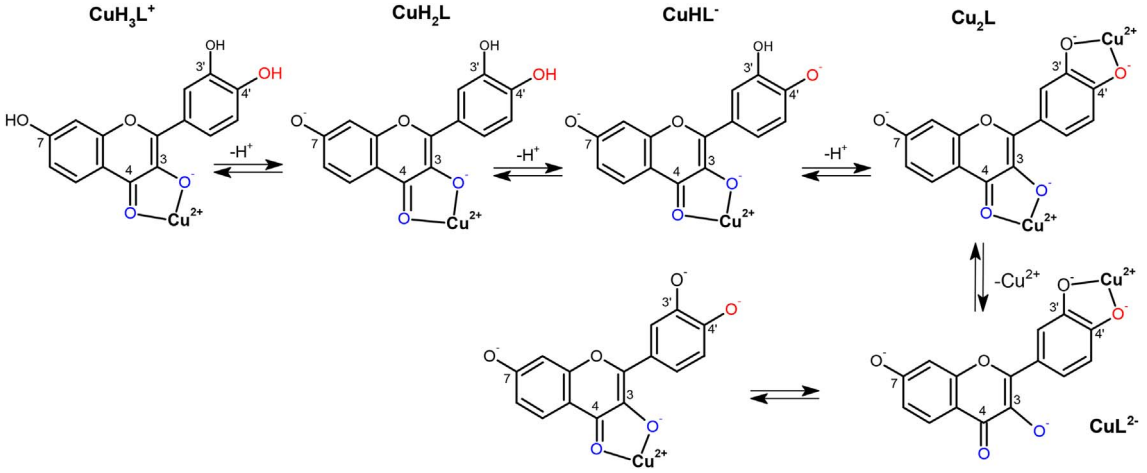


Fig. 4. Proposed pathway of complex formation in the systems containing 1:1 or 2:1 ratios of Cu(II):fisetin.

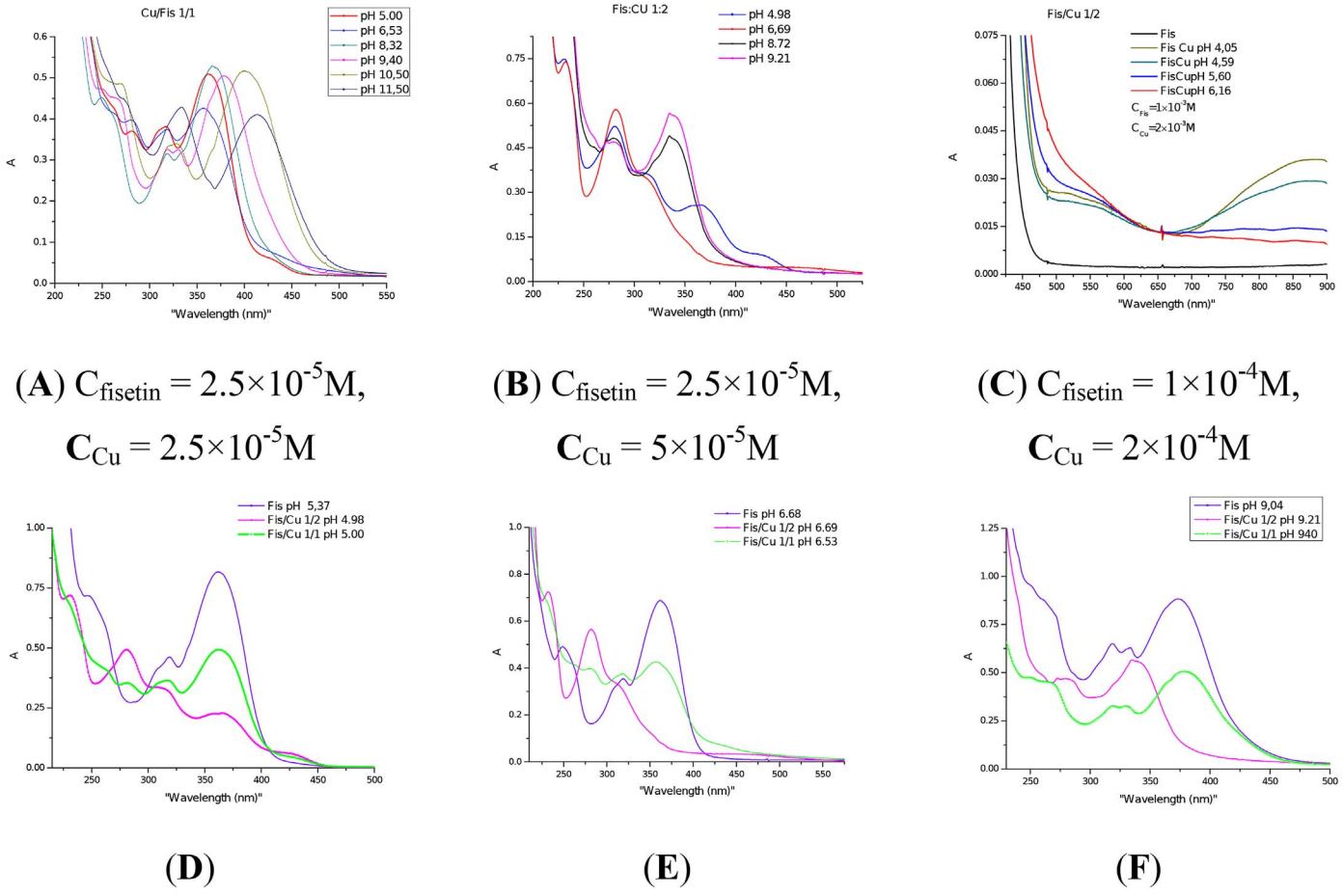


Fig. 5. Titration electronic absorption spectra of Fis–Cu(II) system with ligand to metal molar ratios: 1:1 (A) and 1:2 (B) in pH range 5.00–11.50 or 4.98–9.40, respectively in solution of 40%/60% CH_3OH/water and 0.1 M NaCl. The spectra of complexes and fisetin in acidic, neutral and alkaline pH regions (D), (E), (F).

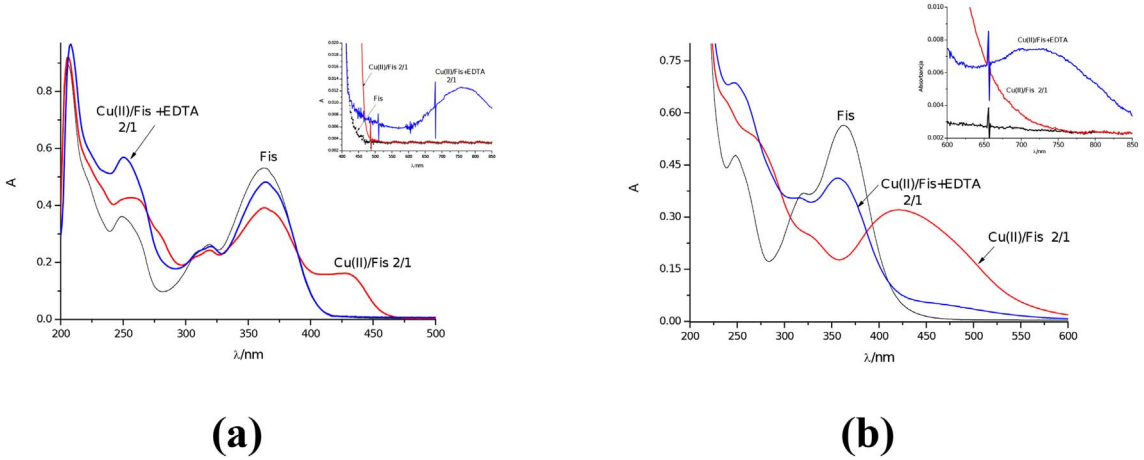
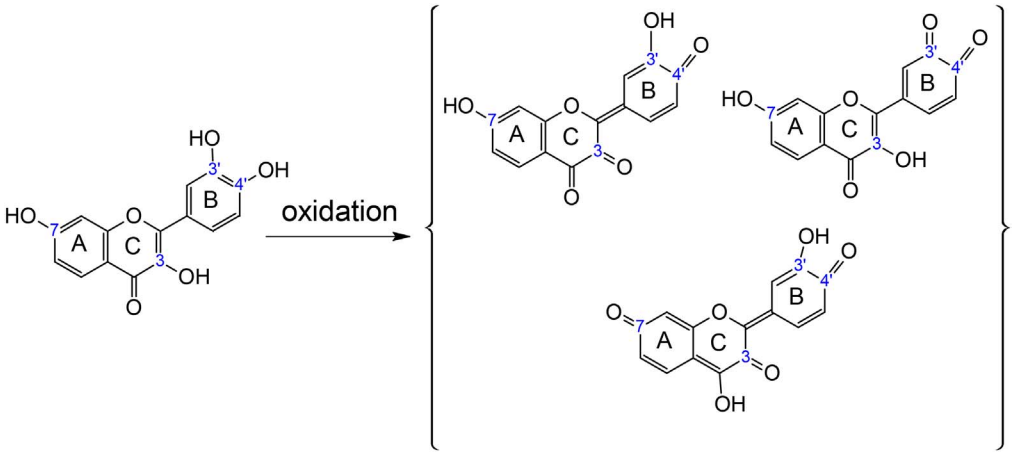


Fig. 6. Effect of EDTA on Cu(II)/fisetin 2/1 system at: (a) pH 3.20 methanol solution; (b) pH 7.00 phosphate buffer in aqueous solution with 1% of methanol. Incubation time 15 min; $C_{fis} = 2.5 \times 10^{-5}$ M, $C_{EDTA} = 1.25 \times 10^{-4}$ M. The insets represent Vis spectra in the range of 400–850 nm.

buffers at pH values between 3.5 ($E_{p,a}$ = 0.37 V) and 8.0 ($E_{p,a}$ = 0.14 V).

The voltammetric response of 5×10^{-5} M solution of fisetin in $LiClO_4$-CH_3OH-H_2O system shows an anodic process at 0.39 V (*vs* SCE) with a backward associated broad wave at about −0.27 V and two further irreproducible waves at higher potential values (between 0.80 and 1.20 V) (Fig. 7 cv1). The results are in accordance with literature data showing that the less anodic process is attributable to the oxidation of the C(3′)— and C(4′)—OH groups on the B-ring of the fisetin, the more anodic processes can be tentatively associated with the oxidation of the OH groups on A and C rings [68]. Voltammetric responses in acetate and phosphate buffers show a similar behavior, with a shift of the first anodic process to lower potential values at pH changing from 3.5 (E_p = 0.39 V) to 8.0 (E_p = 0.14 V). Addition of Cu(II) in a ratio Cu (II):fisetin 2:1 to the $LiClO_4$-CH_3OH-H_2O system (Fig. 7 cv2) leads to a broadening of the first anodic process of the ligand. After the first backward scan, the following anodic scan shows two processes (at 0.00 and 0.15 V, respectively) typically associated with the reoxidation of Cu



Scheme 2. Structures of possible tautomeric quinone forms of fisetin as possible oxidation products of fisetin in solution containing 2-fold excess of Cu(II) ions.

(0) → Cu(I) and Cu(I) → Cu(II), supporting no absorption of Cu(0) on the electrode surface. After 1 h an orange precipitate is formed, very likely due to the presence of a complex scarcely soluble under the experimental conditions. No evidence of free Cu(II) is observed in the voltammetric curves, indicating that fisetin coordinates all the metal in solution, thus forming a 2:1 metal-to-ligand complex. A similar behavior is also showed at pH 7.0 (Fig. 7 cv3). Voltammetric curves indicate the presence of small amount of Cu(II) uncomplexed. It is supported by the appearance of a small peak at about 0.00 V attributable to the process of Cu(0) → Cu(II). At pH 8.0, no evidence of free Cu(II) is observed. On the other hand, a precipitate appears at higher pH values. A different process is evidenced in acetate buffer at pH 3.5 and 4.5; a pale yellow precipitate starts to form after about 1 h. Its colour becomes gradually deeper in few hours. The final voltammetric response (Fig. 7 cv4) shows a sharp reoxidation peak at − 0.10 V, typical for the Cu (0) → Cu(II) process, suggesting the presence of free (uncomplexed) Cu (II) in solution. According to these experimental evidences, we hypothesize that in this case complex of 1:1 stoichiometry is formed. With increasing pH value to pH 5.5, the precipitate is again formed in the first minutes of the reaction. The colour of the precipitate turns to yellow-orange, but a reoxidation sharp peak of Cu(0) to Cu(II) is still present in the voltammogram suggesting the contemporary presence of 2:1 and 1:1 complexes. The cyclic voltammetry results confirm a different mechanism of complexation depending on the pH conditions as it has previously been shown in the species distribution curves (Fig. 3). In particular, CV and species distribution curves indicate that below pH 5.5 uncomplexed Cu(II) ions are always present, with small amounts of complex with Cu(II):fisetin 1:1 ratio. The presence of Cu(II):fisetin 2:1 species is more noticeable at pH 5.5. Finally, the amount of Cu(II) uncomplexed quickly decreases with the pH increase above 5.5, and

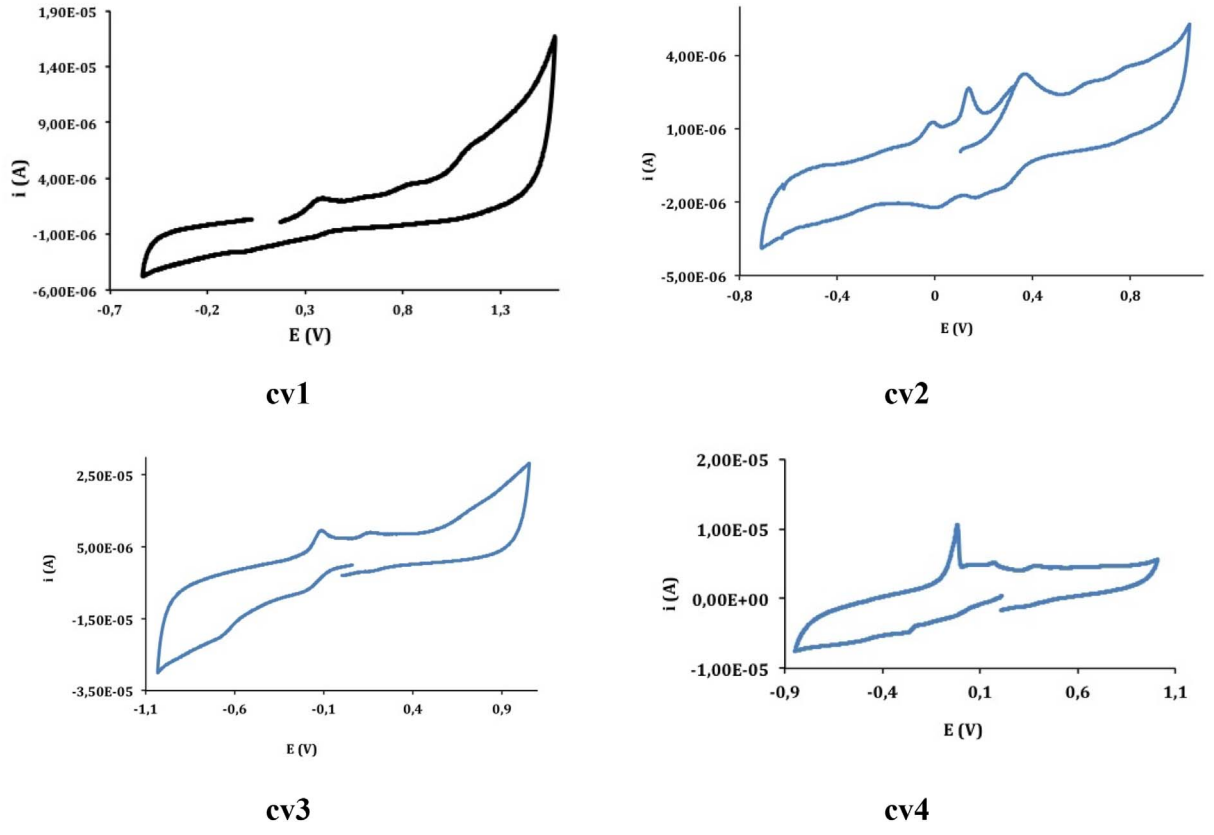


Fig. 7. Cyclic voltammetry responses of fisetin (cv1); M:L 2:1 (cv2); M:L 2:1 in phosphate buffer, pH 7.0 (cv3) and M:L 2: 1 in acetate buffer, pH 3.5 (cv4). All solutions are in CH_3OH/H_2O 40%/60% v/v with 0.1 M $LiClO_4$ as supporting electrolyte. Potential scan rate: 0.1 V s^{-1}; concentration of fisetin: 5×10^{-5} M, copper(II): 1×10^{-4} M.

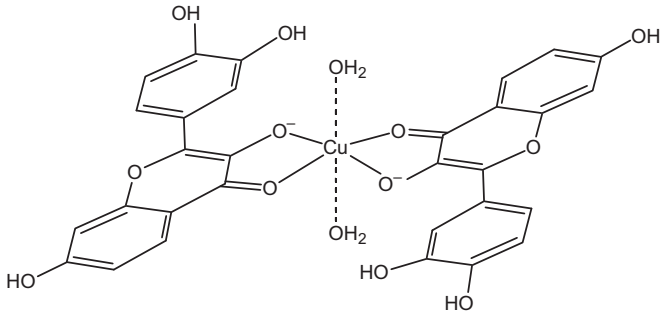
mainly/only the 2:1 complex is formed. Moreover, the voltammetric indications are in good agreement with the results of DPPH test (see the Section 3.3). As it is suggested by the voltammetric data, the coordination of Cu(II) ions generates the changes in electronic structure of the ligand leading to a decrease of its oxidation potential value in the solution containing Cu(II) under the pH range from 3.5 to pH 8.0. The antioxidant activity of flavonoids is highly related to the oxidation potential value, being the activity higher when the potential is lower [69].

As it was pointed out above (Section 3.2) a solid complex is formed in the system of Cu(II) and fisetin with a metal:ligand ratio of 1:2. This complex has been synthesized mixing $CuSO_4{\cdot}5H_2O$ and fisetin in a mixture of CH_3OH/H_2O 40/60 v/v at pH ~ 5; it composition is $[Cu(H_3L)_2(H_2O)_2]{\cdot}3H_2O$ (**1**·3H$_2$O). The FT-IR spectra of the ligand and complex **1** were recorded in the 4000–600 cm^{-1} region. The ν(CO) stretching frequency, which falls at 1638 cm^{-1} in the free ligand, is slightly shifted to 1614 cm^{-1} in the metal complexes indicating the involvement of the carbonyl oxygen in the metal coordination, similarly to what was observed for other transition metal complexes of flavonoids [70–73]. The region between 3600 and 3200 cm^{-1}, attributed to the stretching vibrations of O—H bonds, undergoes significant changes upon complexation but the resolution of the bands is not lost, suggesting that OH groups of the ligand take part to the metal coordination and that water molecules are bound to Cu; in fact, water molecules of crystallization would give only a broad and not resolved absorption. The bending vibration of C—O—C bond does not show significant changes (1276 cm^{-1} in the free ligand *vs* 1282 cm^{-1} in the metal complex), indicating a weak alteration of the structure of ring C and lack of interaction of endocyclic oxygen atom with the Cu(II) ion [62]. The frequency of vibration of the aromatic ring C=C bond is found at 1568 and 1506 cm^{-1} in the ligand and 1566 and 1503 cm^{-1} in the complex **1** [74]. Therefore, on the basis of IR spectroscopy, it can be supposed the binding of the carbonyl CO and of the deprotonated phenolic group O^- in the positions 4 and 3 of the ring C. The electronic absorption spectrum shows only one band attributable to d-d transition, at λ_{max} = 687 nm with ε_{max} = 92 M^{-1} cm^{-1}. This is compatible with an elongated octahedral structure. EPR spectrum of the polycrystalline complex **1** was recorded at 120 K and is shown in Fig. S7. The spectrum is 'tetragonal' with two *g* values in the order: $g_z > g_x = g_y > g_e$; in particular, g_z = 2.211 and > $g_x = g_y$ = 2.070 (Table 3). It indicates a ground state based on $d_{x^2-y^2}$ orbital and axial symmetry, compatible with a square planar or an elongated octahedron species [75–77]. When the solid complex is dissolved in an organic solvents (DMF, DMSO, MeOH), an EPR spectrum due to a mononuclear Cu(II) species is detected and the hyperfine coupling between the unpaired electron and ^{63,65}Cu nuclei is observed (Fig. S8). The values of g_z are in the range 2.303–2.307 and those of A_z in the range 159.1–163.4 × 10^{-4} cm^{-1} (Table 3). These values can be compared with those of $[Cu(acac)_2(H_2O)_2]$, where acac is acetylacetonate, and suggest a CuO_6 coordination with the weak binding of two solvent molecules in the axial position. In fact, a square planar complex should be characterized by a smaller value of g_z and larger of A_z; for example, from $[Cu(acac)_2(H_2O)_2]$ to $[Cu(acac)_2]$ the value of g_z goes from 2.291 to 2.256

Table 3
EPR parameters of the complex **1** in the solid state and in organic solution.

Powder/solvent	g_z	g_x, g_y	$A_z/10^{-4}$ cm^{-1}	Ref.
Powder	2.211	2.070	–	This work
DMF	2.306	[a]	159.1	This work
DMSO	2.307	[a]	159.4	This work
MeOH	2.303	[a]	163.4	This work
$[Cu(acac)_2(H_2O)_2]$[b]	2.291	2.058	175	[78]
$[Cu(ma)_2(H_2O)_2]$[b]	2.314	[a]	164.1	[79]

[a] Value not reported.
[b] ma = maltolate, acac = acetylacetonate.



Scheme 3. Proposed structure of the solid complex **1**.

and that of A_z from 174 × 10^{-4} cm^{-1} to 192 × 10^{-4} cm^{-1} [78]. The spectra recorded in organic solution are almost coincident with that of the bis-chelated complex of maltolate, $[Cu(ma)_2(H_2O)_2]$, g_z = 2.314 and A_z = 164.1 × 10^{-4} cm^{-1} with the two equatorial five-membered chelate rings formed by the donor set (CO, O^-) [79]. Therefore, the solid complex **1** formed by fisetin can be described with the stoichiometry $[Cu(H_3L)_2(H_2O)_2]$ with two H_3L^- ligands equatorially binding copper(II) with the (CO, O^-) donor set in a "maltol-like" coordination, and the weak coordination of two axial water ligands. The structure is shown in Scheme 3.

3.3. Radical scavenging capacity of the systems containing fisetin or fisetin-Cu(II) ions

Total free radical-scavenging capacity was evaluated by spectrophotometrically measuring the disappearance of the free 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The DPPH· test has previously been successfully employed in assessing the antioxidant activity [80]. The studies were performed at the initial concentration of DPPH 5.765 × 10^{-5} M and the concentration of fisetin or complexes ranged from 8 × 10^{-6} to 4 × 10^{-4} M. Influence of Cu(II) ions alone has been also investigated for comparison but no effect on DPPH· radical was found. The data in Fig. 8 show that fisetin and copper complexes can scavenge DPPH· radical in a dose dependent manner. It was found that scavenging capabilities of fisetin and Cu(II)/fisetin 1/2 or Cu(II)/fisetin 1/1 are similar. It indicates that coordination of copper ions in the [C(4)—O_{ket}, C(3)—O^-] site does not affect fisetin capability although at the highest concentration (4 × 10^{-4} M) this capability is slightly

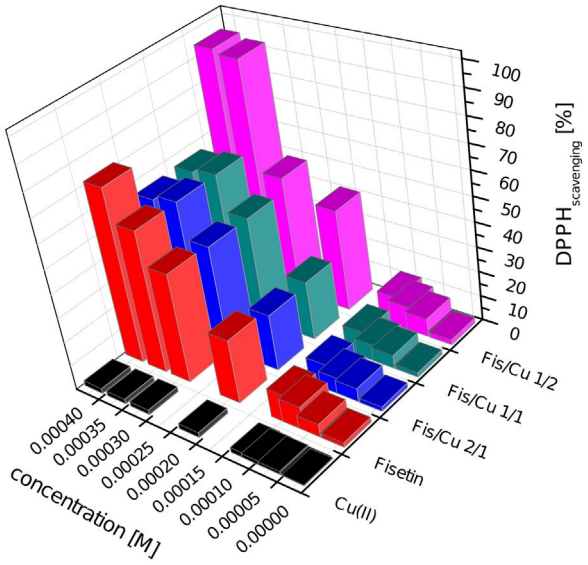
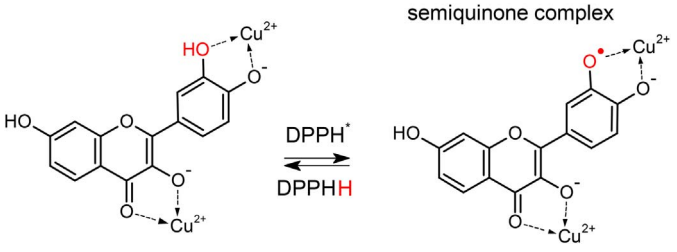


Fig. 8. Percentage decay of DPPH· radical absorption ($DPPH_{scavenging}$) with respect to concentrations of fisetin, complexes and Cu(II) ions.



Scheme 4. Proposed pathway of copper–fisetin complex in scavenging DPPH· radical.

suppressed (Figs. 8, S2). The DPPH· absorbance decays much faster in the presence of double excess of copper ions than for free ligand. It suggests that the radical scavenging activity of the Cu_2(fisetin) complex is greater than fisetin. The *ortho*-dihydroxy structure of the B ring of a flavonoid has the best electron-donating properties and confers higher stability in the radical form and participates in electron delocalization. Similarly, the 2,3-double bond with a 4-oxo function in the C ring, are responsible for electron delocalization from the B ring. After binding copper(II) ion by two oxygen atoms derived from the first coordination site of [C(4)—O_{ket}, C(3)—O^-] the electron delocalization is very likely stabilized and the scavenging radical capability is similar to fisetin [81]. Possible pathway in the case of Cu_2(fisetin) complex could be as that proposed in Scheme 4. A hydrogen atom may be abstracted from the [C(3′)—OH, C(4′)—O^-] chelating site to give a semiquinone stronger stabilized by the metallic centers than in fisetin or Cu(fisetin) complex. Similar effect was observed for Cu_2(quercetin) complex [82]. After the complexation with two metal ions, the scavenging properties increase probably due to the presence of positively charged meal ions as well as electron donating groups present in the moiety, so complexes have strong potential to be applied as scavengers to eliminate radicals [52,83].

Total antioxidant activity of the compounds was also studied by using $ABTS^+$· radical cation assay. Scavenging radical capability was determined by following the decolourisation (reduction) of $ABTS^+$· radical cation for various concentrations of fisetin, complexes and Trolox. The suppression of the absorbance of $ABTS^+$· in a concentration-dependent manner was observed (Fig. S9). The $ABTS^+$· radical scavenging activity of the compounds was higher in comparison to that of the reference compound Trolox. The test complexes showed lower radical scavenging efficiency than fisetin at lower concentrations but at higher concentrations they were more effective and similar to fisetin in quenching $ABTS^+$· radicals. Due to the fact that, the scavenging of $ABTS^+$· is assumed to be an electron transfer process: $ABTS^+$· + e^- → ABTS [84] we can suppose that at high concentrations a more efficient transfer of electrons between $ABTS^+$· radicals and the complexes occurs. Our results indicate that fisetin and complexes can effectively inhibit both DPPH· and $ABTS^+$· although some differences of scavenging efficiency of the radicals are observed. It may suggest that these compounds exert radical-scavenging action in different pathway, very likely by donating hydrogen atom (H·) and/or electron (e^-).

3.4. Mitochondrial studies

Mitochondria play a key role in multiple cellular functions [85,86]. In the past decade, the field of mitochondria biology has been focused on the dynamic and interactive features of these semiautonomous organelles. There is interest in determining whether antioxidants will reduce mitochondria oxidative damage and prevent the appearance of oxidative stress. Some polyphenols are recognized as molecules capable of modulating pathways that define intra-mitochondrial oxidative status *i.e.*, inhibiting/inducing of ROS, formation/removal enzymes *inter alia* induction of endogenous antioxidant-synthesizing enzymes *e.g.*, glutathione synthase [87]. *In vitro* studies of oxidative stress effects in mitochondria are useful models to disclose the mechanisms of oxidative cell injury, and to evaluate the sensitivity of cellular components to oxidative metabolic stress [88,89]. Investigation of antioxidant enzyme levels could be informative way about antioxidant action. The present study was designed to evaluate antioxidant activity of fisetin (H_4L/H_3L) or its solid complex ($[Cu(H_3L)_2(H_2O)_2]$) on the oxidative effects of tBHP (*tert*-butylhydroperoxide) induced in rat liver mitochondria. The results are reported in Table 4. The tBHP leads to a significant decrease in the reduced form of glutathione (GSH) (75%), increase in the content of the mixed glutathione-protein disulfide (GSSP) (3 times) and the level of lipid peroxidation products (TBARS) (3 times), which is characterized by impaired antioxidant defense system and damage mitochondrial membranes. Total thiols (TSH) and protein thiols (PSH) were not significantly changed under tBHP action but in the presence of the complex statistically significant increase of TSH and PSH can be observed. One of the possible reasons behind the elevated quantities of TSH and PSH might be that the complex could regenerate the protein thiols in contact with protein disulfides within the mitochondrial matrix [90,91]. Moreover, the level increment of TSH could be caused by recovery, for example, of cysteine from its disulfide form or GSH from GSSG [34]. Both glutathionylation and the formation of intra-protein disulfides can dramatically affect the activity of enzymes and transcription factors, enabling them to respond reversibly to the ambient GSH/GSSG ratio, just as proteins are regulated by reversible phosphorylation [90]. The level of lipid peroxidation products (TBARS) induced by tBHP increased 3,5 times ($p < 0.05$) compared to the control. The fisetin prevented elevation of TBARS levels by 25–50%. The complex reduced TBARS level at the highest extent in comparison with tBHP (Table 4). Regarding the enzymatic activity, it can be inferred that *tert*-butyl hydroperoxide at a concentration of 1.5 mM has no effect on enzyme activities of glutathione transferase (mtGT) or glutathione peroxidase (mtGPx), which play a crucial role in antioxidant and enzymatic activities of mitochondria. Fisetin and the complex have an activating effect on mtGT in comparison to tBHP. The activating effect of fisetin is also observed for mtGPx while the copper

Table 4
Protein sulfhydryl groups (PSH), reduced glutathione (GSH), mixed glutathione-protein disulfides (GSSP), lipid peroxidation products (TBARS), glutathione transferase (mtGT) and glutathione peroxidase (mtGPx) levels (expressed as nmol/mg protein) in rat liver cell mitochondria.

Parameters [nmol/mg protein]	Compounds [average ± SD]			
	Control	tBHP	Fis	$[Cu(H_3L)_2(H_2O)_2]$·
TSH	91.40 ± 2.46	83.94 ± 1.21	85.57 ± 4.32	102.46 ± 5,9
GSH	14.04 ± 0.68	3.57 ± 0.214[1]	3.47 ± 0.19[1]	3.90 ± 0.2[1]
PSH	77.36 ± 1.52	80.37 ± 2.35	82.10 ± 4.21	92.85 ± 5.9[1]
TBARS	0.028 ± 0.003	0.084 ± 0.004[1]	0.063 ± 0.009[1]	0.041 ± 0.008[2]
GSSP	0.487 ± 0.035	1.562 ± 0.099[1]	1.535 ± 0.019[1]	1.32 ± 0,08[1]
mtGT	26.72 ± 0.5	25.00 ± 2.5	32.52 ± 0.9**#	37.69 ± 3,8[1]
mtGPx	522.3 ± 74.7	460.5 ± 102.4	766.0 ± 40.8[2]	458.4 ± 29.2

[1] Statistically significant in comparison with control, $p < 0.05$.
[2] Statistically significant in comparison with tBHP, $p < 0.05$.

complex has a very slight effect on the mtGPx level comparing to tBHP (Table 4). Our results suggest that fisetin or its copper complex can behave as pro-oxidants by increasing mitochondrial level of TSH, GSH, PSH, mtGT and decreasing level of GSH or as antioxidants by decreasing level of TEBARS and partially restoring the content of reduced glutathione in the case of the complex. The pro-oxidant activity would be advantageous when the anticancer and apoptosis induction properties of fisetin or copper complex are considered, since reactive oxygen species can mediate apoptotic DNA fragmentation [92–95].

3.5. DNA binding studies

Nowadays, many studies focus on the design of compounds that have the potential for binding to DNA. DNA is the primary intracellular target of an anticancer compound because the interaction between these molecules can block the division of cancer cells and resulting in cell death. UV–Vis electron absorption spectroscopy, fluorimetry and circular dichroism were applied to find a clue about DNA binding with fisetin and copper complexes.

UV–Vis absorption spectroscopy was applied firstly as one of the most used methods for investigating the effects of any compound on DNA. The binding abilities of fisetin and copper complexes with stoichiometries: 1/1, 1/2, 2/1 (Fis/Cu) have been characterized through absorbance and the shift in the wavelength as a function of added concentration of CT DNA [Fig. 9]. Upon addition of increasing amount of CT DNA to fisetin-Tris solution, a significant hypochromism along with blue shift of about 30 nm with respect to the fisetin band at λ_{max} 361 nm is observed. This can be attributed to the interaction between DNA and compound and it is also likely that the compound can bind to the DNA *via* intercalation. The blue shift may be due to H-bonding interactions between intercalating ligand and the base pair of the DNA. The appearance of isosbestic point in the spectrum may indicate that: (i) the compound binds to DNA in a single mode, (ii) the presence of a new species formed during the interaction, (iii) it enables the assumption of two-state system consisting of bound and free fisetin species in the binding process that are in equilibrium [96,97]. In the spectra of all complexes there is a strong band at $\lambda_{max} \approx 400$ nm attributed to $\pi \rightarrow \pi^*$ transitions [98]. This absorption band is significantly changed upon addition of increasing amount of CT DNA and hypochromic and bathochromic effects are observed in the spectra. The extent of hypochromism and bathochromism can be correlated with an intercalative binding strength. The plots of $[DNA]/(\varepsilon_a - \varepsilon_f)$ *vs* [DNA] for absorption titration of the studied compounds, useful for obtaining K_b by the ratio of the slope to the intercept are reported in (Fig. 9(b)). The K_b values were obtained for Fis $(3.59 \pm 0.15) \times 10^4$, and the systems with the ratios of Fis/Cu 1/1, 1/2 and 2/1 respectively as follows $(3.52 \pm 0.18) \times 10^4$, $(3.71 \pm 0.17) \times 10^4$, $(4.13 \pm 0.17) \times 10^4$ (M^{-1}). Upon complexation no significant increase of K_b is observed with respect to the ligand, only slightly higher value of K_b is seen for the complex with Fis/Cu stoichiometry 2/1. The electronic spectrum of this complex shows the presence of a shoulder (at ≈475 nm) not visible in the spectra of the other compounds, which highlights the different behavior and suggests a presence of a new molecular species responsible for interactions. Our experimental K_b values are lower than those observed for classical intercalators (ethidium-DNA, 3.0×10^6 in 5 mM Tris-HCl/50 mM NaCl buffer, pH = 7.2 [99] indicating that the compounds bind DNA with less affinity, as already noticed for copper (II) complexes with macrocyclic ligands [100]. Nevertheless, for the studied compounds, a partial intercalation cannot be excluded to explain the K_b values, or an external mode of binding to DNA leading to modest electronic coupling with the host, as it had been already noted for Cu(II) complexes [101]. To verify better and confirm these results, fluorescence quenching experiments and circular dichroism spectra were made.

For further investigation one of complex with 2:1 Fis/Cu) stoichiometry was chosen due to its relatively better affinity to DNA as absorption titration's results indicated. Fluorescence spectroscopy was used in order to get a deeper insight into fisetin or copper complex interaction with CT DNA. The possible mode of their binding can be obtained from the ethidium bromide displacement assay. Ethidium bromide (EB) is a well-known classical DNA intercalator and a standard fluorescent tag. If any molecule can displace EB, then the fluorescence intensity of EB-DNA adduct decreases suggesting intercalation of the molecule inside the helix [102,103]. Thus the extent of fluorescence quenching of EB bound to CT DNA can be used to determine the extent of binding between the second molecule and CT DNA [104–106]. A gradual decrease in the fluorescence intensity occurs on addition of the complex in EB-DNA adduct, as depicted in Fig. S10 (in SI). The addition of complex to DNA pretreated with EB showed appreciable reduction in the emission intensity. On the addition of 20 μM complex to 25 μM of CT DNA pretreated with EB, *ca.* 65% decrease in intensity of EB-DNA adduct was observed (Fig. S11). The quenching of EB bound to CT DNA by the complex is in good agreement with the linear Stern–Volmer equation ($I_0/I = 1 + K_{sv}[C]$) which provides further evidence that the complex binds to DNA. The value of Stern–Volmer quenching constant (K_{sv}) was 9.71(± 0.04) × 10^4 (R^2 = 0.9882). The apparent binding constant, K_{app} of the studied complex was calculated from the equation: $K_{app,complex} \times [complex] = K_{app,EB} \times [EB]$, where $K_{app,EB}$ is the apparent binding constant of EB assumed to be $1 \times 10^7\ M^{-1}$ [107]. The value of $K_{app,complex}$ is 8.0×10^6. The results obtained for both K_{SV} and K_{app} show that the complex can compete for DNA binding sites and so displace EB from the DNA, which is usually characteristic of the intercalative interaction of compounds with DNA [108].

The changes in conformation of DNA due to its complexation both with fisetin and the complex with 2:1 Fis/Cu stoichiometry as $[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$ (in short $(Fis)_2Cu$) were monitored by circular dichroism study. Circular dichroism (CD) is a useful technique to assess whether nucleic acids undergo conformational changes as a result of complex formation or changes in the environment [109]. The DNA helix has a right-hand chiral structure, maintaining the B conformation in solution. The CD absorption spectrum is very sensitive to conformational changes in DNA [110]. The circular dichroism spectra for the DNA–Fis and DNA–$(Fis)_2Cu$ interactions in the region of 210–400 nm are presented in Fig. 10. The CD spectra of CT-DNA show a positive band at 275 nm and a negative band at 245 nm, due to base stacking and right-hand helicity, respectively, consistent with the characteristic B conformation of DNA [111,112]. Spectral analysis of DNA–Fis system indicates small changes in intensity of both bands for ratios of DNA/fisetin: 2/1 and 1/1. It can only be observed slight red shift (≈1 nm) in the band at λ_{max} 276 nm. On the other hand the spectrum registered with a double excess of fisetin relatively to DNA points out significant changes in DNA conformation. These alterations in the DNA CD spectrum may be attributed to both intercalation effects and/or to the formation of DNA–fisetin adduct. The influence of $(Fis)_2Cu$ complex on the conformation of the DNA molecule is seen in Fig. 10(B). It was observed a significant change in both bands of DNA for all studied ratios of DNA/$(Fis)_2Cu$ as compared to the ligand which actually provides an additional support to our previous observation noted from other techniques. The extent of perturbation of conformation of DNA was critical for a double excess of the complex with respect to CT DNA. In this case a decay of the both bands at 245 and 275 nm can be seen. Such an impact of $(Fis)_2Cu$ complex could be a result of its strong interactions with DNA leading to disruption of the biopolymer structure. It can provide a conclusion that not only partial intercalative pathway but very likely oxidative strand breakage could occur as a result of reducing properties of fisetin with respect to Cu(II) ions [113,114]. Fenton-like reactions with involvement of Cu(I) ions may lead to generate ROS species capable of damaging DNA structure.

3.6. DNA cleavage studies

The ability of fisetin and its solid complex ($[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$)

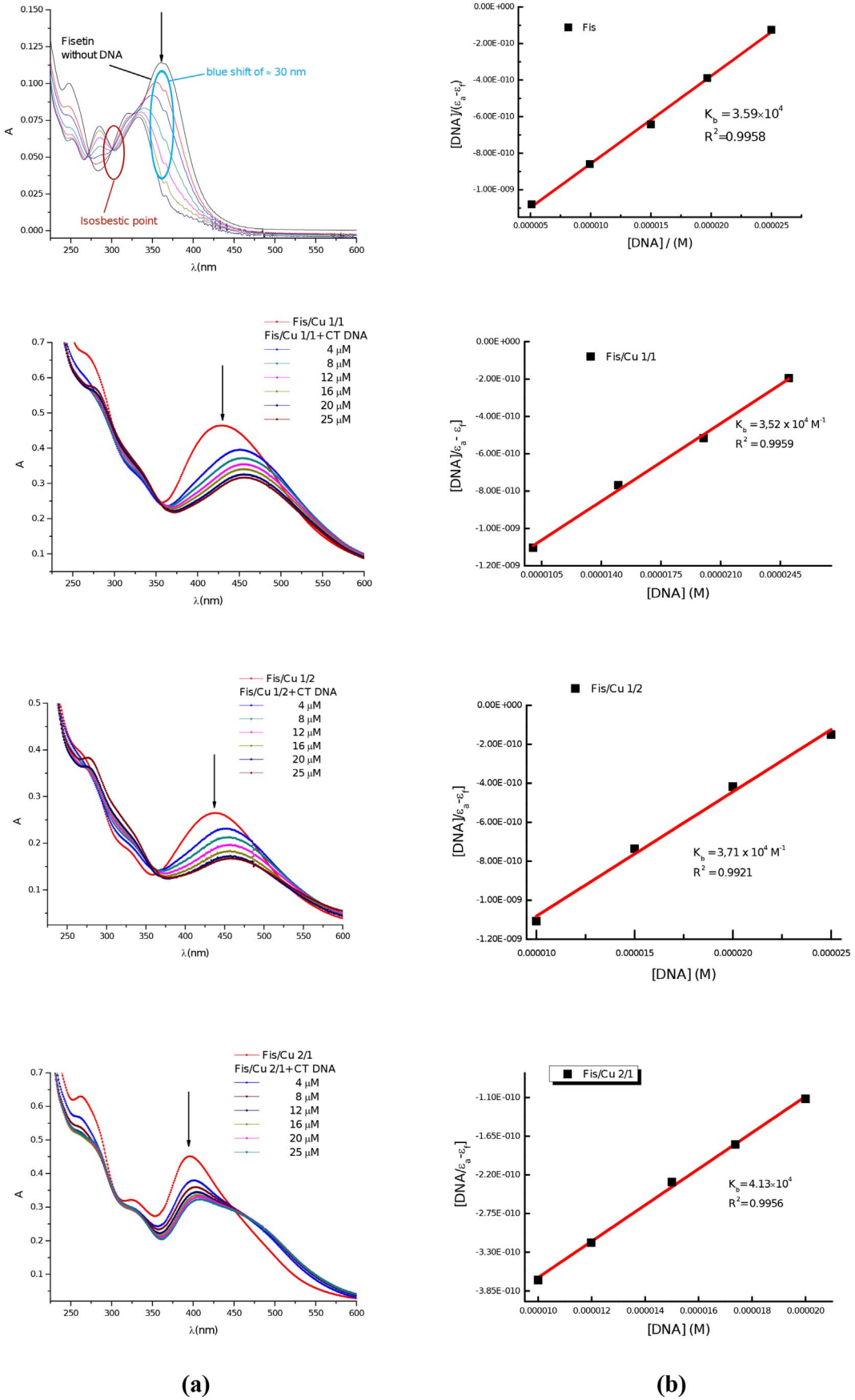


Fig. 9. (a) Absorption spectra of 1×10^{-5} M fisetin and fisetin/Cu(II) systems at different ratios in the absence and in the presence of CT DNA from 0 to 25 μM. Incubation time 30 min. The arrow direction indicates increasing concentration of CT DNA. (b) Plots of $[DNA]/(\varepsilon_a - \varepsilon_f)$ *vs* [DNA] for absorption titration of the studied compounds.

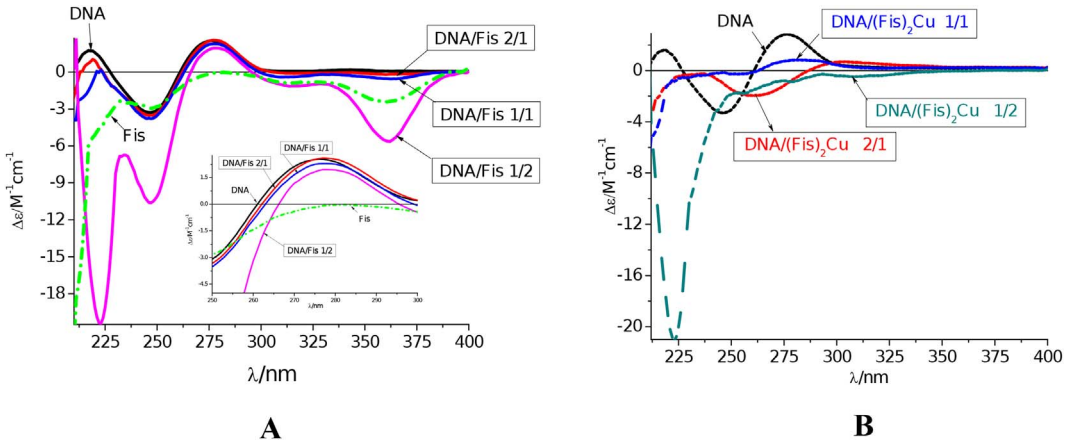


Fig. 10. (A) CD spectra of DNA with fisetin at different ratios of DNA/Fis: 2/1, 1/1, 1/2; $C_{DNA} = C_{fis} = 9.4 \times 10^{-5}$ M in TRIS buffer. The insert represents the same spectra in the range of 250–300 nm. (B) CD spectra of DNA and the $(Fis)_2Cu$ complex at different ratios of DNA/complex: 2/1, 1/1, 1/2. $C_{DNA} = C_{complex} = 9.4 \times 10^{-5}$ M in TRIS buffer.

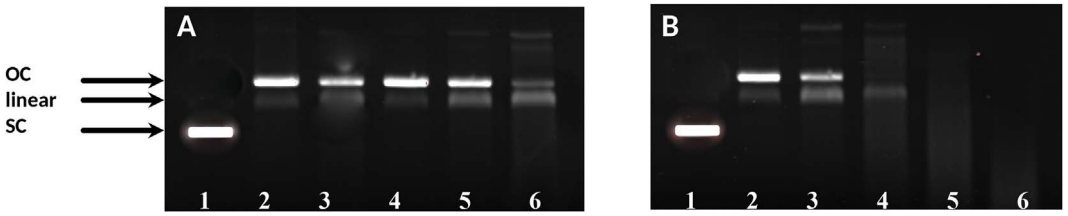


Fig. 11. Agarose gel electrophoresis pattern for the cleavage of pEGFP-C1 plasmid DNA by fisetin (A) and the copper complex ($[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$) (B) at 37 °C after incubation for 10 h at different concentrations of fisetin: 1–DNA control, 2–25 μM + DNA, 3–50 μM + DNA, 4–100 μM + DNA, 5–150 μM + DNA, 6–200 μM + DNA and the complex **1**–DNA control, 2–2.5 μM + DNA, 3–5.0 μM + DNA, 4–10.0 μM + DNA, 5–15.0 μM + DNA, 6–20.0 μM + DNA.

to cleave DNA was studied by incubating them with supercoiled (SC) pEGFP-C1 in the absence of any activator in 5 mM Tris-HCl/5 mM NaCl buffer at pH 7.5 for 10 h at 37 °C. The plasmid can exist in fast relaxed supercoiled Form I and two low migration rate forms namely, singly nicked relaxed circular Form II and doubly nicked linear Form III [115]. The concentration dependent DNA cleavage activity of the compounds was observed by gel electrophoresis (Fig. 11). With increase in concentration of fisetin or the complex, the amount of Form I of pEGFP-C1 plasmid DNA gradually diminishes whereas Form II and Form III increases, suggesting the single or double strand DNA cleavage. At 50 μM concentration and above, fisetin exhibited efficient nuclease activity, the supercoiled form (Form I) relaxes to generate Form II and III. In the case of the complex there was a complete conversion of Form I into Form II at 2.5 μM concentration while at 5 μM was observed Form III. Above this last concentration a degradation of plasmid DNA was clearly evident. The cleavage patterns reveal a higher nucleolytic efficiency of complex in comparison to fisetin which corroborates well with the DNA binding studies (see *supra*) and the literature reports regarding DNA breakage mediated by copper-flavonoid's complexes [116].

3.7. Antimicrobial activities

Influence of fisetin, copper complex ($[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$) and copper(II) ions derived from $CuCl_2$ against 8 Gram-positive, 2 Gram-negative strains of bacteria and 4 strains of fungi was determined as percentage of growth inhibition (Table 5). Copper ions were included in the studies because they mediate bactericidal mechanisms leading to damages of bacteria. The changes were observed at minimal concentration 0.2 μM of the tested compounds. As positive controls were vancomycin, ampicillin for bacteria and nystatin for fungi strains. The susceptibility of different species to Cu(II), complex and fisetin clearly varies as it can be observed from Table 5. The complex reveals antifungal activity for all strains of fungi while fisetin only for *Aspergillus ochraceus*. Likewise, it has stronger antibacterial effect almost for all strains of bacteria. There is one exceptional case namely *Salmonella* Typhimurium ATCC 14028 which is susceptible to Cu(II) ions but not to the complex or the ligand. Fisetin can act only against three Gram-positive strains of bacteria *Listeria monocytogenes* ATCC 19115, *Staphylococcus epidermidis* ATCC 12288 and *Staphylococcus epidermidis* PCM 2480. On the other hand Cu(II) ions do not reveal any antagonistic activity against *Staphylococcus epidermidis* ATCC 12288. Antagonistic activity of *Staphylococcus epidermidis* PCM 2480 was shown as an example in Fig. S12. Increase of autoaggregation percentage was noticeable under the complex and fisetin for two bacteria strains (Table 6). The aggregation of cells can decrease their access to oxygen and facilitate the accumulation of waste products to prevent proliferation and induce the initiation of cell death [117]. We may hypothesize that the hydrophobic sites (pockets) of proteins present at membrane cells of bacteria may provide a possible hosting site for molecules. Moreover, fisetin–copper complex may function as molecular “fastener” that penetrates into the hydrophobic sites of membranes or proteins and initiate their adhesion and aggregation. Therefore we could observe a stronger growth inhibition for bacteria in the presence of the complex. Furthermore, the complex can function as a metal–ionophore delivering copper ions into cells. It could lead to the generation of oxidative stress but also from the high affinity of the Cu(II) Lewis acid for amino acid side chains such as that of cysteine or histidine. It likely results in misfolded proteins or the displacement of other transition metal cations from native active sites [118]. These processes may participate in cell–cell interaction and protein clusterization and may provide protection against infection and disease. Regarding the results one can conclude that sensitivity and susceptibility of an organism to tested compounds varies considerably among different species and also between populations of one single strain depending on physiological state of the culture as it was the case in other similar studies [119,120]. The antimicrobial data suggest that both ligand and complex are biologically active to a greater or less extent, and can act as agents inhibiting the growth of bacteria and fungi.

Table 5
Antagonistic activity of tested compounds at a concentration of 0.2 μM against selected bacteria and fungi.

Test strain	Growth inhibition zone [mm]						
	C	FC	F	DMSO	Vancomycin 0.021 μM	Ampicillin 0.006 μM	Nystatin 0.022 mM
Listeria monocytogenes ATCC 19115	7.3 ± 0.47[a]	16.3 ± 1.70[b]	9.3 ± 2.1[ca]	0.0 ± 0.0	20.3 ± 0.47	11.7 ± 0.47	nd
Listeria monocytogenes ATCC 19111	5.3 ± 0.47[a]	12.0 ± 0.82[b]	0.0 ± 0.0[c]	0.0 ± 0.0	20.0 ± 0.82	16.0 ± 0.82	nd
Staphylococcus aureus ATCC 27734	3.3 ± 0.47[a]	8.7 ± 0.94[b]	0.0 ± 0.0[c]	0.0 ± 0.0	14.0 ± 0.81	18.0 ± 2.22	nd
Staphylococcus aureus ATCC 25753	2.0 ± 0.0[a]	6.7 ± 0.47[b]	0.0 ± 0.0[c]	0.0 ± 0.0	15.3 ± 0.47	19.3 ± 0.47	nd
Staphylococcus epidermidis ATCC 12288	0.0 ± 0.0[a]	10.3 ± 0.47[b]	2.3 ± 0.47[c]	0.0 ± 0.0	16.0 ± 0.82	19.3 ± 0.47	nd
Staphylococcus epidermidis PCM 2480	7.3 ± 0.47[a]	12.3 ± 0.47[b]	4.7 ± 0.94[c]	0.0 ± 0.0	15.0 ± 0.00	20.0 ± 0.81	nd
Enterococcus faecalis ATCC 29212	6.0 ± 0.81[a]	6.3 ± 0.94[a]	0.0 ± 0.0[b]	0.0 ± 0.0	15.0 ± 0.00	20.0 ± 0.00	nd
Enterococcus faecalis ATCC 51299	3.6 ± 0.47[a]	9.3 ± 0.47[b]	0.0 ± 0.0[c]	0.0 ± 0.0	5.3 ± 1.24	14.3 ± 0.47	nd
Salmonella Typhimurium ATCC 14028	3.0 ± 0.00[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0	0.0 ± 0.0	7.60 ± 0.94	nd
Salmonella Enteritidis ATCC 1307	3.0 ± 0.00[a]	8.7 ± 2.35[b]	0.0 ± 0.0[c]	0.0 ± 0.0	0.0 ± 0.0	9.0 ± 0.0	nd
Geotrichum candidum ŁOCK 0511	1.7 ± 0.21[a]	3.7 ± 0.47[b]	0.0 ± 0.0[c]	0.0 ± 0.0	nd	nd	3.0 ± 0.47
Alternaria alternata ŁOCK 0409	1.8 ± 0.24[a]	2.7 ± 0.47[a]	0.0 ± 0.0[b]	0.0 ± 0.0	nd	nd	8.7 ± 1.24
Aspergillus ochraceus	0.0 ± 0.0[a]	5.2 ± 0.24[b]	2.5 ± 0.41[c]	0.0 ± 0.0	nd	nd	3.7 ± 1.7
Penicillium spp.	0.0 ± 0.0[a]	3.3 ± 0.47[b]	0.0 ± 0.0[a]	0.0 ± 0.0	nd	nd	5.5 ± 0.41

C – $CuCl_2$; FC – complex ($[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$); F – fisetin; nd – not detection; [a,b,c]statistical differences between the investigated compounds for the microorganism, $p \leq 0.05$.

Table 6
Autoaggregation of test bacteria after 5 hour incubation.

Strain of bacteria	Autoaggregation [%] ± SD			
	C	FC	F	Control
Salmonella Typhimurium ATCC 14028	6.0 ± 0.36[a]	52.4 ± 6.29[b]	82.0 ± 14.85[c]	15.3 ± 2.25[d]
Salmonella Enteritidis ATCC 1307	10.1 ± 1.20[a]	45.9 ± 9.25[b]	80.8 ± 16.28[c]	22.1 ± 8.21[a]

C – $CuCl_2$; FC – complex ($[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$); F –fisetin; [a,b,c]statistical differences between the investigated compounds for the microorganism, $p \leq 0.05$.

4. Conclusions

The formation and stability of copper-fisetin complexes were systematically investigated in the present studies. Although the formation of flavonoid-transition metal complexes is quite complicated, our results showed that using potentiometric, voltammetric and spectroscopic methods these complexes can be detected in solution with high sensitivity. We confirmed the chelating potency of fisetin in a wide pH range especially at acidic conditions. The results lead to the conclusion that, at neutral pH there is no clearly defined ionization state of fisetin or the complex molecules and different neutral and ionized forms can coexist. This may affect the mechanism of biological activity of this flavonol or its complexes *in vivo*.

Antioxidant activity of fisetin or complexes depends on their concentration. High concentrations promote distinctly their radical scavenging effects. An analysis of the DPPH free radical antioxidant properties reveals that the complexes exhibit a greater quenching effect than fisetin itself whereas in the reaction with the ABTS radical, the fisetin shows a greater antioxidant effect compared to the complexes. The lower antioxidant activity of copper complexes may be due to their higher oxidative potential compared to the free ligand, which may play a greater role in the case of the ABTS than in DPPH radical. This suggests that metal ions significantly alter the physicochemical properties of the ligand due to deprotonation of hydroxyl groups and formation of chelating sites for the metal ions. Results of mitochondrial studies indicate that fisetin or its copper complex can behave as pro-oxidants by increasing mitochondrial level of TSH, GSH, PSH, mtGT and decreasing level of GSH or as antioxidants by decreasing level of TEBARS and partially restoring the content of reduced glutathione in the case of the complex. However, it should be mentioned that the net effects of fisetin or copper complex could be different in different tissues, and only an overall analysis based on *in vivo* studies can determine the physiological significance of the effects of these compounds for the whole organism.

The binding properties with calf thymus DNA revealed that fisetin and its Cu(II) complex bind to DNA by partially intercalation and/or oxidative modes. The results obtained from all spectroscopic studies indicate the stronger damaging/nucleating activity of Fis/Cu(II) 2/1 complex. The reason could be that the ligand can possess a greater density of negative charges than the copper complex, which may prevent its strong interaction with DNA duplex as compared to the complex especially at low concentration. Finally, both fisetin and its copper complex have been found to promote cleavage of plasmid pEGFP-C1 DNA in the absence of external agent. It should be stressed the importance of the dose level of fisetin as a component of nutritional supplements because of its possible damaging effect on cellular DNA especially at high concentration.

The results of antimicrobial studies lead to the conclusion that both fisetin and the copper complex ($[Cu(H_3L)_2(H_2O)_2]\cdot 3H_2O$) display different antimicrobial activities against tested strains of bacteria and fungi. Differences may be due to properties of the bacteria or fungi related to structure, functional groups and surface area, depending on the bacterial or fungal division, genera and species. Antibacterial activities of the ligand and its complex compared with those of the standard drugs are lower. The most promising results have been obtained with respect to an antagonistic activity of the complex towards the studied fungi. It inhibited the growth of fungi at similar extent as the standard drug - nystatin but at 110 times lower concentration. Concluding we may say that toxic effect of fisetin or the complex in bacteria or fungi is affected by their structure and physiology, and cannot be assumed to be due to a single universal mechanism. Our studies may contribute to a better understanding of the chelating chemistry of flavonoids with copper ions and beyond and further promote the research in the discovery of compounds with anticancer and antimicrobial activities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2017.12.006.

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